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Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors

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SUMMARY

The reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) raises the possibility that a somatic cell could be reprogrammed to an alternative differentiated fate without first becoming a stem/progenitor cell. A large pool of fibroblasts exists in the postnatal heart, yet no single "master regulator" of direct cardiac reprogramming has been identified. Here, we report that a combination of three developmental transcription factors (i.e., Gata4, Mef2c, and Tbx5) rapidly and efficiently reprogrammed postnatal cardiac or dermal fibroblasts directly into differentiated cardiomyocyte-like cells. Induced cardiomyocytes expressed cardiac-specific markers, had a global gene expression profile similar to cardiomyocytes, and contracted spontaneously. Fibroblasts transplanted into mouse hearts one day after transduction of the three factors also differentiated into cardiomyocyte-like cells. We believe these findings demonstrate that functional cardiomyocytes can be directly reprogrammed from differentiated somatic cells by defined factors. Reprogramming of endogenous or explanted fibroblasts might provide a source of cardiomyocytes for regenerative approaches.

INTRODUCTION

Heart disease is a leading cause of adult and childhood mortality. The underlying pathology is typically loss of cardiomyocytes that leads to heart failure or improper development of cardiomyocytes during embryogenesis that leads to congenital heart malformations. Because postnatal cardiomyocytes have little or no regenerative capacity, current therapeutic approaches are limited. Embryonic stem cells possess clear cardiogenic potential, but efficiency of cardiac differentiation, risk of tumor formation, and issues of cellular rejection must be overcome (Ivey and Srivastava, 2006; Laflamme et al., 2007; Nussbaum et al., 2007; van Laake et al., 2008). The ability to reprogram fibroblasts into induced pluripotent stem cells (iPSCs) with four defined factors might address some of these issues by providing an alternative source of embryonic-like stem cells (Takahashi and Yamanaka, 2006). However, generating sufficient iPSC-derived cardiomyocytes that are pure and mature and that can be delivered safely remains challenging (Zhang et al., 2009).

The human heart is composed of cardiomyocytes, vascular cells, and cardiac fibroblasts. In fact, cardiac fibroblasts comprise over 50% of all the cells in the heart (Baudino et al., 2006; Camelliti et al., 2005; Snider et al., 2009). Cardiac fibroblasts are fully differentiated somatic cells that provide support structure, secrete signals, and contribute to scar formation upon cardiac damage (Ieda et al., 2009). Fibroblasts arise from an extracardiac source of cells known as the proepicardium, and do not normally have cardiogenic potential (Snider et al., 2009). The large population of endogenous cardiac fibroblasts is a potential source of cardiomyocytes for regenerative therapy if it were possible to directly reprogram the resident fibroblasts into beating cardiomyocytes. Unfortunately, although embryonic mesoderm can be induced to differentiate into cardiomyocytes (Takeuchi and Bruneau, 2009), efforts to accomplish this in somatic cells have thus far been unsuccessful, and to our knowledge, no "master regulator" of cardiac differentiation, like MyoD for skeletal muscle (Davis et al., 1987), has been identified to date.

The generation of iPSCs suggests that a specific combination of defined factors, rather than a single factor, could epigenetically alter the global gene expression of a cell and allow greater plasticity of cell type than previously appreciated. Consistent

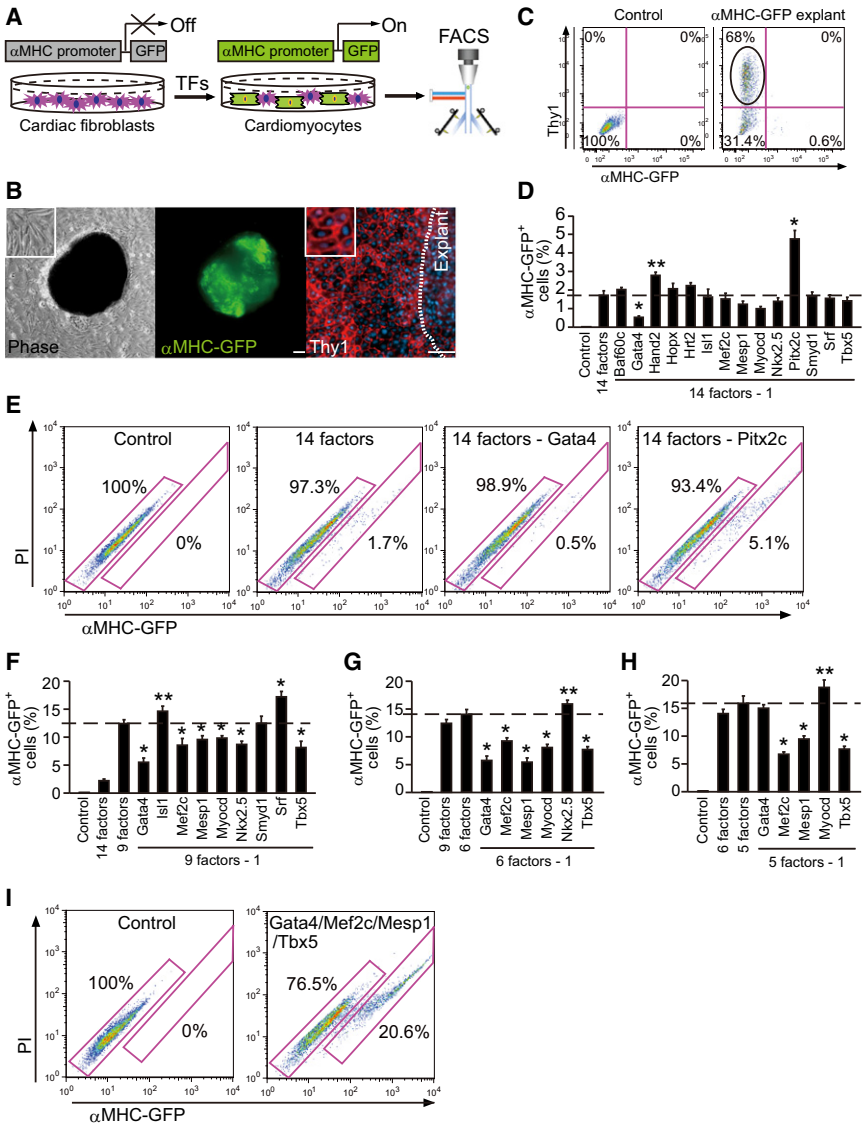

Figure 1. Screening for Cardiomyocyte-Inducing Factors

(A) Schematic representation of the strategy to test candidate cardiomyocyte-inducing factors.

(B) Morphology and characterization of fibroblast-like cells migrating from αMHC-GFP heart explants. Phase contrast (left), GFP (middle), and Thy-1 immunostaining (right). Insets are high-magnification views. See also Figure S1.

(C) Thy-1$^+$/GFP$^-$ cells were FACS sorted from explant cultures for reprogramming.

(D) Summary of FACS analyses for α-MHC-GFP$^+$ cells. Effect on GFP$^+$ cell induction with 14 factors or the removal of individual factors from the pool of 14 factors (n = 3). Removal of Baf60c, Hand2, Hopx, Hrt2, or Pitx2c did not decrease the percent of GFP$^+$ cells and were excluded for further analyses. See also Figure S2.

(E) FACS plots for analyses of GFP$^+$ cells. GFP$^+$ cells were analyzed 1 week after 14 factor transduction. The number of GFP$^+$ cells were reduced by removal of Gata4, but increased by removal of Pitx2c from 14 factors.

(F–H) Effect on GFP$^+$ cell induction of the removal of individual factors from the pool of 9 (F), 6 (G), or 5 (H) factors (n = 3). Factors that did not decrease efficiency upon removal were excluded from further study.

(I) GFP$^+$ (20%) cells were induced from fibroblasts by the combination of four factors, Gata4, Mef2c, Mesp1, and Tbx5. Representative data are shown in each panel. PI, propidium iodine. All data are presented as means ± SD. *$p < 0.01$; **$p < 0.05$ versus relevant control. Scale bars represent 100 μm. See also Figures S1 and S2.

with this, the bHLH transcription factor, Neurogenin 3, in combination with Pdx1 and Mafa, can efficiently reprogram pancreatic exocrine cells into functional β cells in vivo, although the exocrine cells were known to have some potential to become islet cells in vitro and share a common parent cell with islet cells (Baeyens et al., 2005; Zhou et al., 2008). A combination of three factors, Ascl1, Brn2, and Myt1l, converts dermal fibroblasts to functional neurons (Vierbuchen et al., 2010), although the degree of global reprogramming of the neurons is unknown.

In this study, we examined whether key developmental cardiac regulators could reprogram cardiac fibroblasts into cardiomyocytes. We found that out of a total of 14 factors, a specific combination of three transcription factors, Gata4, Mef2c, and Tbx5, was sufficient to generate functional beating cardiomyocytes directly from mouse postnatal cardiac or dermal fibroblasts and that the induced cardiomyocytes (iCMs) were globally reprogrammed to adopt a cardiomyocyte-like gene expression profile.

RESULTS

Screening for Cardiomyocyte-Inducing Factors

We developed an assay system in which the induction of mature cardiomyocytes from fibroblasts could be analyzed quantitatively by reporter-based fluorescence-activated cell sorting (FACS) (Figure 1A). To accomplish this, we generated αMHC promoter-driven EGFP-IRES-puromycin transgenic mice (αMHC-GFP), in which only mature cardiomyocytes expressed the green fluorescent protein (GFP) (Gulick et al., 1991). We confirmed that only cardiomyocytes, but not other cell types such as cardiac fibroblasts, expressed GFP in the transgenic mouse hearts and in primary cultured neonatal mouse cardiac cells (Figure S1 available online).

To have enough cardiac fibroblasts for FACS screening, we obtained GFP$^-$ cardiac fibroblasts from neonatal αMHC-GFP hearts by explant culture. Fibroblast-like cells migrated from the explants after 2 days and were confluent after 1 week. The migrating cells did not express GFP, but expressed Thy1 and vimentin, markers of cardiac fibroblasts (Figure 1B and data not shown) (Hudon-David et al., 2007; Ieda et al., 2009). To avoid contamination of cardiomyocytes, we filtered the cells by cell

strainers to remove heart tissue fragments and isolated Thy1$^+$/GFP$^-$ cells by FACS (Figure 1C). Using FACS, we confirmed that Thy1$^+$/GFP$^-$ cells did not express cardiac troponin T (cTnT), a specific sarcomeric marker of differentiated mature cardiomyocytes (Figure S1) (Kattman et al., 2006). With these procedures, we had no cardiomyocyte contamination in the fibroblast culture and could generate greater than twice the number of cardiac fibroblasts than by conventional fibroblast isolation techniques (Ieda et al., 2009).

To select potential cardiac reprogramming factors, we used microarray analyses to identify transcription factors and epigenetic remodeling factors with greater expression in mouse cardiomyocytes than in cardiac fibroblasts at embryonic day 12.5 (Ieda et al., 2009). Among them, we selected 13 factors that exhibited severe developmental cardiac defects and embryonic lethality when mutated (Figure S2). We also included the cardiovascular mesoderm-specific transcription factor Mesp1 because of its cardiac transdifferentiation effect in *Xenopus* (David et al., 2008). We generated individual retroviruses to efficiently express each gene in cardiac fibroblasts (Figure S2).

We transduced Thy1$^+$/GFP$^-$ neonatal mouse cardiac fibroblasts with a mixture of retroviruses expressing all 14 factors or with DsRed retrovirus (negative control) (Hong et al., 2009). We did not observe any GFP$^+$ cells in cardiac fibroblasts 1 week after Ds-Red retrovirus infection or 1 week of culture without any viral infection. In contrast, transduction of all 14 factors into fibroblasts resulted in the generation of a small number of GFP$^+$ cells (1.7%), indicating the successful activation of the cardiac-enriched α*MHC* gene in some cells (Figures 1D and 1E).

To determine which of the 14 factors were critical for activating cardiac gene expression, we serially removed individual factors from the pool of 14. Pools lacking five factors (Baf60c, Hand2, Hopx, Hrt2, and Pitx2c) produced an increased number of GFP$^+$ cells, suggesting they are dispensable in this setting (Figures 1D and 1E). Of note, removing Gata4 decreased the percentage of GFP$^+$ cells to 0.5%, and removing Pitx2c increased it to 5%. Removal of the five factors listed above resulted in an increase in the percentage of GFP$^+$ cells to 13% (Figure 1F). We conducted three further rounds of withdrawing single factors from nine-, six-, and five-factor pools, removing those that did not decrease efficiency upon withdrawal, and found that four factors (Gata4, Mef2c, Mesp1, and Tbx5) were sufficient for efficient GFP$^+$ cell induction from cardiac fibroblasts (Figures 1F–1H). The combination of these four factors dramatically increased the number of fibroblasts activating the α*MHC*-GFP reporter to over 20% (Figure 1I).

Gata4, Mef2c, and Tbx5 Are Sufficient for Cardiomyocyte Induction

Next, we examined the expression of cTnT by FACS. We found that 20% of GFP$^+$ cells expressed cTnT at high enough levels to detect by FACS 1 week after the four-factor transduction. Again removing individual factors from the four-factor pool in transduction, we found that Mesp1 was dispensable for cTnT expression (Figures 2A and 2B). In contrast, we did not observe cTnT$^+$ or GFP$^+$ cells, when either Mef2c or Tbx5 was removed. Removal of Gata4 did not significantly affect the number of GFP$^+$ cells, but cTnT expression was abolished, suggesting Gata4 was also required. Whereas the combination of two factors, Mef2c and Tbx5, induced GFP expression but not cTnT, no combination of two factors or single factor induced both GFP and cTnT expression in cardiac fibroblasts (Figure 2C). These data suggested that the combination of three factors, Gata4, Mef2c, and Tbx5, is sufficient to induce cardiac gene expression in fibroblasts.

We found that 30% of GFP$^+$ cells expressed cTnT 1 week after the three-factor transduction. Next, to confirm our screening results, we transduced cardiac fibroblasts with three factors (Gata4, Mef2c, and Tbx5, hereafter referred to as GMT) plus Nkx2-5, a critical factor for cardiogenesis but excluded by our initial screening. Surprisingly, adding Nkx2-5 to GMT dramatically inhibited the expression of GFP and cTnT in cardiac fibroblasts. We also transduced cardiac fibroblasts with the combination of Baf60c, Gata4, and Tbx5, which can transdifferentiate noncardiogenic mesoderm to cardiomyocytes in mouse embryos (Takeuchi and Bruneau, 2009). We found that this combination did not efficiently induce cTnT or GFP expression above that of Tbx5 alone, confirming our screening results (Figure 2D).

To determine if other cardiac genes were enriched in GFP$^+$ cells, we sorted GFP$^+$ cells and GFP$^-$ cells 7 days after transduction with GMT and compared gene expression of cardiomyocyte-specific genes, *Myh6* (α-myosin heavy chain), *Actc1* (cardiac α-actin), *Actn2* (actinin α2), and *Nppa* (natriuretic peptide precursor type A) by quantitative RT-PCR (qPCR). We found that these cardiac genes were upregulated significantly more in GFP$^+$ than in GFP$^-$ cells (Figure 2E). Next, we used immunocytochemistry to determine if cardiac proteins were expressed in GFP$^+$ cells. Despite the detection of cTnT in only 30% of GFP$^+$ cells, most GFP$^+$ cells induced with the three factors expressed sarcomeric α-actinin (α-actinin) and had well-defined sarcomeric structures, similar to neonatal cardiomyocytes (Figure 2F; Figure S1). In addition to α-actinin, some GFP$^+$ cells also expressed cTnT and ANF (atrial natriuretic factor), indicating GFP$^+$ cells expressed several cardiomyocyte-specific markers (Figures 2G and 2H). We also confirmed that neither GFP$^+$ nor GFP$^-$ cells expressed smooth muscle or endothelial cell markers (Figure S2), suggesting specificity of GMT effects.

Induced Cardiomyocytes Originate from Differentiated Fibroblasts and Are Directly Reprogrammed

We next isolated neonatal cardiac fibroblasts by the conventional fibroblast isolation method in which hearts were digested with trypsin and plated on plastic dishes (Ieda et al., 2009). More than 85% of the cells expressed Thy1, and we isolated Thy1$^+$/GFP$^-$ cells by FACS to exclude cardiomyocyte contamination (Figure 3A). Fibroblasts transduced with GMT expressed GFP, cTnT, and actinin after 1 week at the same level as fibroblasts isolated from explant cultures (Figures 3B and 3C). Similar results were obtained on introduction of GMT into adult cardiac fibroblasts, with full formation of sarcomeric structures (Figure 3D; Figure S2).

To determine if the induced cardiomyocyte-like cells (iCMs) were arising from a subpopulation of stem-like cells, we

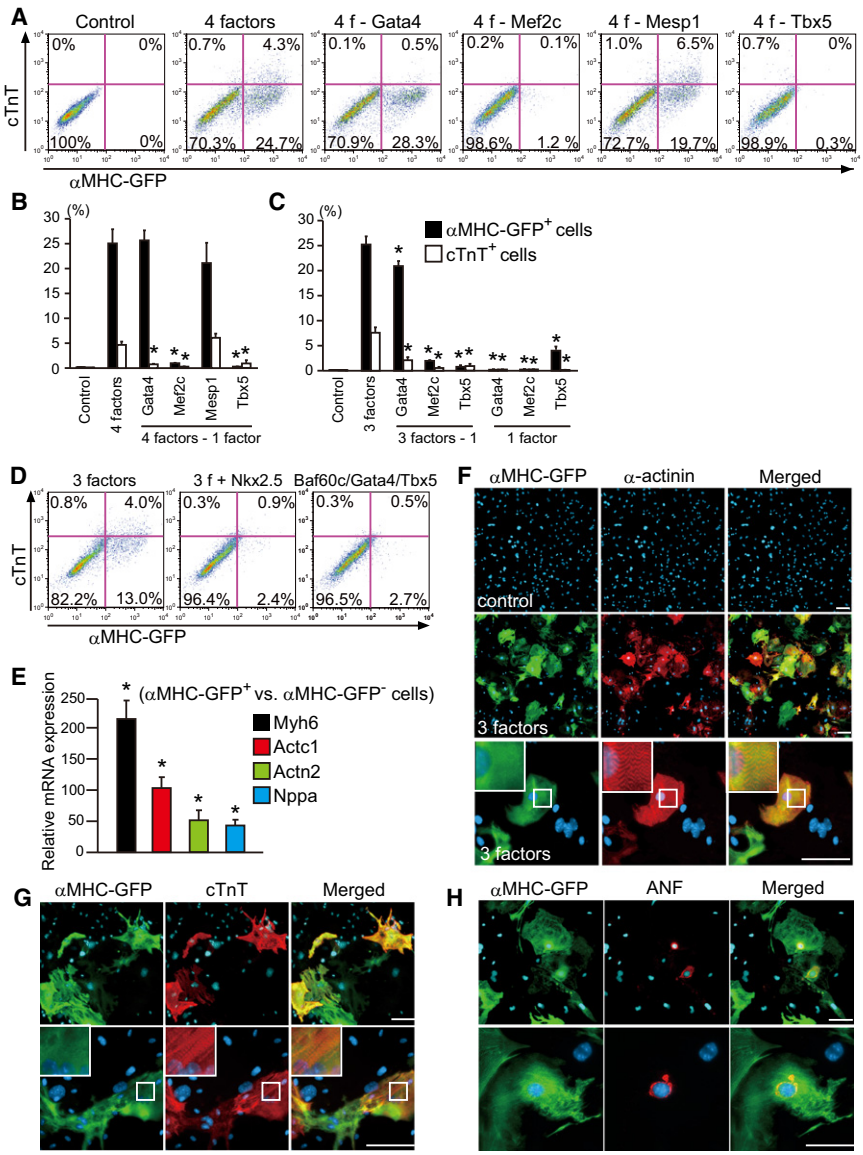

Figure 2. Combination of Three Transcription Factors Induces Cardiac Gene Expression in Fibroblasts

(A) FACS analyses for α-MHC-GFP and cardiac Troponin T (cTnT) expression. Effects of the removal of individual factors from the pool of four factors on GFP$^+$ and cTnT$^+$ cell induction.

(B) Quantitative data of GFP$^+$ cells and cTnT$^+$ cells in (A) (n = 3).

(C) Effect of the transduction of pools of three, two, and one factors on GFP$^+$ and cTnT$^+$ cell induction (n = 3).

(D) FACS analyses for α-MHC-GFP and cTnT expression. Effects of GMT plus Nkx2.5 and Baf60c/Gata4/Tbx5 transduction are shown.

(E) The mRNA expression in GFP$^+$ and GFP$^-$ cells 7 days after GMT transduction was determined by qPCR (n = 3).

(F) Immunofluorescent staining for GFP, α-actinin, and DAPI. The combination of the three factors, GMT, induced abundant GFP, and α-actinin expression in cardiac fibroblasts 2 weeks after transduction. High-magnification views in insets show sarcomeric organization. See also Figures S1 and S2.

(G) Induced cardiomyocytes expressed cTnT by immunocytochemistry with clear sarcomeric organization 4 weeks after transduction. Insets are high-magnification views.

(H) Induced cardiomyocytes expressed ANF at perinuclear sites 2 weeks after transduction. All data are presented as means ± SD. *p < 0.01 versus relevant control. Scale bars represent 100 μm.

See also Figures S1 and S2.

analyzed c-kit expression (Beltrami et al., 2003) in the Thy1$^+$/GFP$^-$ cells. Most c-kit$^+$ cells coexpressed Thy1, whereas 15% of Thy1$^+$ cells expressed c-kit, which is consistent with a previous report of cardiac explant-derived cells (Davis et al., 2009). We isolated GFP$^-$/Thy1$^+$/c-kit$^+$ cells and GFP$^-$/Thy1$^+$/c-kit$^-$ cells by FACS and transduced each population of cells with GMT. We found 2–3-fold more cardiomyocyte induction in GFP$^-$/Thy1$^+$/c-kit$^-$ cells than in GFP$^-$/Thy1$^+$/c-kit$^+$ cells (Figure S3). These results suggest that most of the iCMs originated from a c-kit-negative population.

We then sought to more definitively exclude the possibility of rare cardiac progenitors giving rise to iCMs. We tested the potential of mouse tail-tip dermal fibroblasts to generate iCMs. We found that sorted Thy1$^+$/GFP$^-$ tail-tip dermal fibroblasts transduced with GMT expressed GFP at the same level as GMT-transduced cardiac fibroblasts, although the percentage of cTnT$^+$ cells was less than cardiac fibroblast-derived iCMs (Figures 3E–3G). Like cardiac fibroblasts, tail-tip fibroblast-derived GFP$^+$ cells expressed α-actinin and had well-defined sarcomeric structures (Figure 3H; Figure S3), suggesting noncardiac fibroblasts can also be reprogrammed into cardiomyocytes by GMT induction. These results excluded the possibility that the iCMs arose from contamination of cardiomyocytes or cardiac progenitors before cardiac induction in the fibroblast population.

We also determined whether the reprogramming of fibroblasts to differentiated cardiomyocytes was a direct event or if the fibroblasts first passed through a cardiac progenitor cell fate before further differentiation. To distinguish between these two possibilities, we used mice expressing Isl1–yellow fluorescent protein (YFP) obtained by crossing Isl1-Cre mice and R26R-EYFP mice (Srinivas et al., 2001) (Figure S3). Isl1 is an early cardiac progenitor marker that is transiently expressed before cardiac differentiation. If iCMs generated from fibroblasts passed through a cardiac progenitor state, they and their descendants would permanently express YFP (Laugwitz et al., 2005). We isolated Isl1-YFP$^-$/Thy1$^+$cells from Isl1-YFP heart explants by FACS and transduced the cells with GMT. The resulting cTnT$^+$ cells did not express YFP in significant numbers,

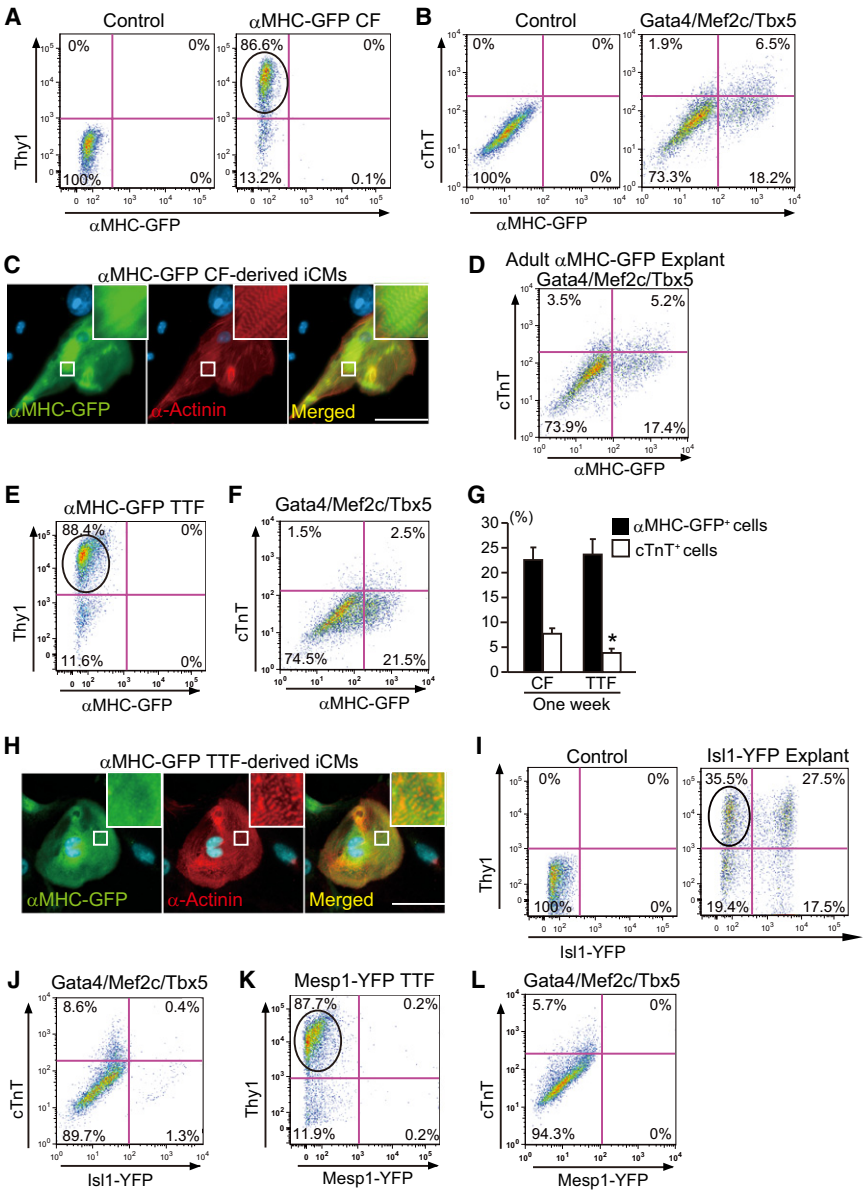

Figure 3. Induced Cardiomyocytes Originate from Differentiated Fibroblasts and Are Directly Reprogrammed

(A) Cardiac fibroblasts (CF) isolated by the conventional isolation method. Most cells were positive for Thy1, and Thy-1$^+$/GFP$^-$ cells were sorted by FACS for transduction.

(B) FACS analyses for αMHC-GFP and cTnT expression in cardiac fibroblasts isolated in (A) 1 week after transduction by GMT.

(C) Immunofluorescent staining for GFP, α-actinin, and DAPI in the GMT induced cardiomyocytes originated from (A).

(D) Cardiac fibroblasts isolated from adult αMHC-GFP hearts were transduced with three factors. See also Figure S2.

(E) Thy-1$^+$/GFP$^-$ tail-tip dermal fibroblasts (TTFs) were sorted by FACS for transduction.

(F) FACS analyses for GFP and cTnT expression in TTFs isolated in (E) 1 week after GMT transduction.

(G) Quantitative data of GFP$^+$ cells and cTnT$^+$ cells indicated in (F) (n = 3 in each group).

(H) Immunofluorescent staining for GFP, α-actinin, and DAPI in TTF-derived iCMs. See also Figure S3.

(I) Isl1-YFP$^-$/Thy1$^+$cells were sorted from Isl1-Cre/Rosa-YFP heart explants and transduced with GMT. See also Figure S3.

(J) The vast majority of cTnT$^+$ cells induced from Isl1-YFP$^-$/Thy1$^+$cells was negative for YFP.

(K) Mesp1-YFP$^-$/Thy1$^+$cells were sorted from Mesp1-Cre/Rosa-YFP TTFs and transduced with GMT. See also Figure S3.

(L) All cTnT$^+$ cells induced from Mesp1-YFP$^-$/Thy1$^+$cells were negative for YFP.

All data are presented as means ± SD. *$p < 0.01$ versus relevant control. Scale bars represent 100 μm. See also Figure S3 for analyses of c-kit$^+$ cells.

See also Figures S2 and S3.

suggesting that the iCMs were not first reprogrammed into Isl1$^+$ cardiac progenitor cells (Figures 3I and 3J). Moreover, these results provided supportive evidence that the iCMs did not originate from a rare population of cardiac progenitor cells that might exist in neonatal hearts.

Whereas Isl1 marks most early cardiac progenitors, a subpopulation of cardiac progenitors remains Isl1 negative. Mesp1 is the earliest pan-cardiovascular progenitor cell marker that is transiently expressed in nascent mesoderm before further cardiovascular differentiation (Figure S3) (Saga et al., 1999). We therefore generated Mesp1-YFP mice by crossing Mesp1-Cre and R26R-EYFP mice to determine if iCMs were reprogrammed into early cardiac mesoderm before further differentiation. We isolated Mesp1-YFP$^-$/Thy1$^+$ tail-tip dermal fibroblasts by FACS and transduced the cells with GMT (Figures 3K and 3L). The resulting cTnT$^+$ cells did not express YFP, suggesting that the iCMs were not converted into the cardiac mesoderm cell state for reprogramming, but rather they were directly reprogrammed into differentiated cardiomyocytes by the three factors (Figure 3L).

Induced Cardiomyocytes Resemble Postnatal Cardiomyocytes in Global Gene Expression

We next analyzed the time course of cardiomyocyte induction from cardiac fibroblasts. GFP$^+$ cells were detected 3 days after induction and gradually increased in number up to 20% at day 10 and were still present after 4 weeks (Figure 4A). GFP$^+$ cells were less proliferative than GFP$^-$ cells and, over time, decreased in percentage relative to the total number of cells. Importantly, the percentage of cTnT$^+$ cells among the α-MHC-GFP$^+$ iCMs and the intensity of cTnT expression increased significantly over time (Figures 4B and 4C). We sorted GFP$^+$ cells at 1, 2, and 4 weeks after transduction with GMT and compared cardiac gene

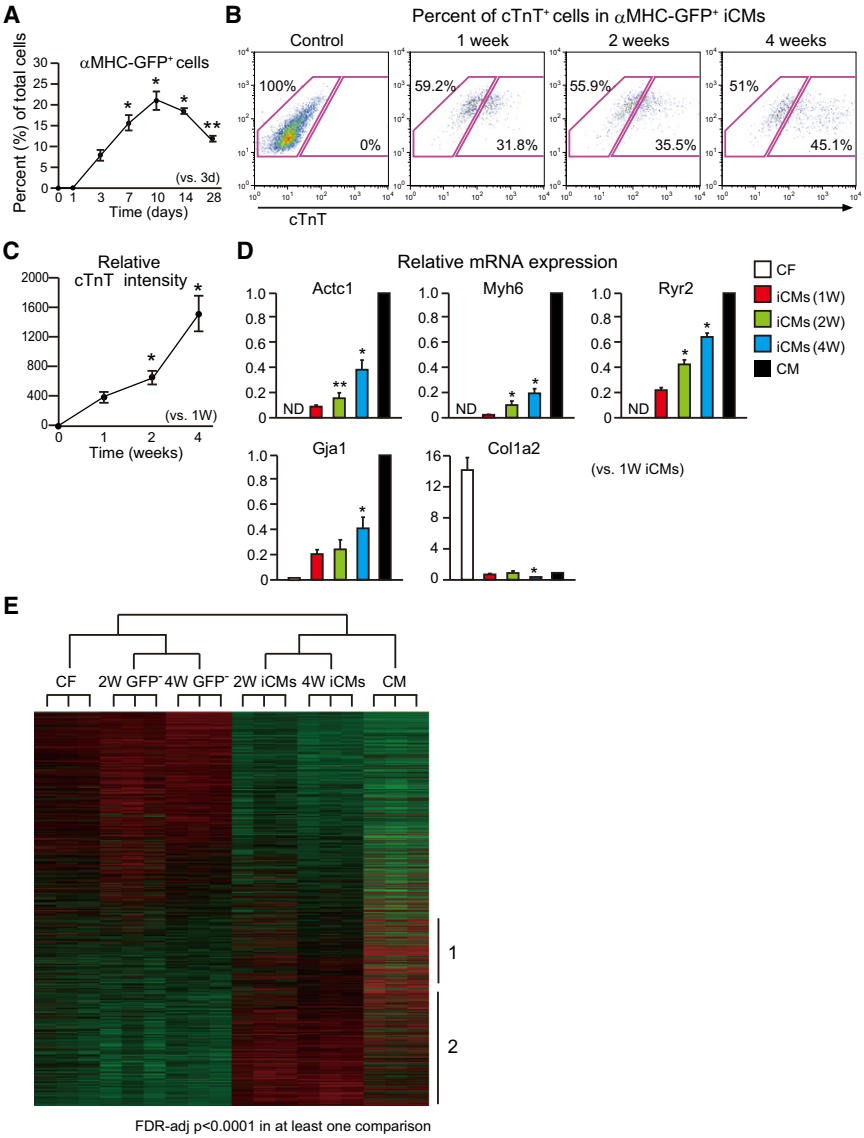

Figure 4. Gene Expression of Induced Cardiomyocytes Is Globally Reprogrammed

(A) The percent of αMHC-GFP$^+$ cells after GMT transduction (n = 3). The number of GFP$^+$ cells was counted by FACS at each time point and divided by the number of plated cells.

(B) FACS analyses of percent of cells with cTnT expression among αMHC-GFP$^+$ iCMs. Note that cTnT $^+$ cell number and cTnT intensity were both increased over time (n = 4).

(C) Quantitative data of cTnT intensity in (B) (n = 4).

(D) *Actc1, Myh6, Ryr2, Gja1*, and *Col1a2* mRNA expression in cardiac fibroblasts (CF), induced cardiomyocytes (iCMs) (1 week (W), 2 W, 4 W after transduction), and neonatal cardiomyocytes (CM), determined by qPCR (n = 3).

(E) Heatmap image of microarray data illustrating differentially expressed genes among CF, α-MHC-GFP$^-$, iCMs (FACS sorted 2 and 4 weeks after transduction), and CM (n = 3 in each group). The scale extends from 0.25- to 4-fold over mean (−2 to +2 in log2 scale). Red indicates increased expression, whereas green indicates decreased expression. Group 1 includes the genes upregulated only in CM, and group 2 includes the genes upregulated in CM and 4W-iCMs compared to CF. Lists of genes are shown in Table S1 and Table S2. All data are presented as means ± SD. *p < 0.01; **p < 0.05 versus relevant control. See also Figure S4 for endogenous and exogenous expression of reprogramming factors and Table S1 and Table S2 for differentially expressed genes.

See also Tables S1 and S2 and Figure S4.

expression with cardiac fibroblasts and neonatal cardiomyocytes. The cardiomyocyte-specific genes, *Actc1*, *Myh6*, *Ryr2* (ryanodine receptor 2), and *Gja1* (connexin43), were significantly upregulated in a time-dependent manner in GFP$^+$ cells, but were not detected in cardiac fibroblasts by qPCR (Figure 4D). *Col1a2* (collagen 1a2), a marker of fibroblasts, was dramatically downregulated in GFP$^+$ cells from 7-day culture to the same level as in cardiomyocytes. These data indicated that the three factors induced direct conversion of cardiac fibroblasts to cardiomyocytes rapidly and efficiently, but full maturation was a slow process that occurred over several weeks. Total gene expression of the three reprogramming factors was upregulated 6- to 8-fold in iCMs over neonatal cardiomyocytes. However, only endogenous expression of *Gata4* was upregulated in iCMs to the same level as in neonatal cardiomyocytes, whereas endogenous *Mef2c* and *Tbx5* expression was lower in iCMs than in cardiomyocytes, potentially reflecting negative autoregulatory loops (Figure S4).

We next compared the progressive global gene expression pattern of iCMs, neonatal cardiomyocytes, and cardiac fibroblasts by mRNA microarray analyses. We sorted GFP$^+$ cells and GFP$^-$ cells 2 and 4 weeks after GMT transduction. The iCMs at both stages were similar to neonatal cardiomyocytes, but were distinct from GFP$^-$ cells and cardiac fibroblasts in global gene expression pattern (Figure 4E). We found that functionally important cardiac genes were upregulated significantly more in 4 week iCMs than in 2 week iCMs, including *Pln* (phospholamban), *Slc8a1* (sodium/calcium exchanger), *Myh6*, *Sema3a* (semaphorin 3a), *Id2* (inhibitor of DNA binding 2), and *Myl2* (myosin, light polypeptide 2, regulatory, cardiac, slow, also known as MLC2v) (Table S1). Conversely, some genes were downregulated more in 4 week iCMs than in 2 week iCMs (Table S1). The array analyses also identified genes that were upregulated more in neonatal cardiomyocytes than in 4 week iCMs or cardiac fibroblasts (group 1 in Figure 4E), including *Bmp10* (bone morphogenetic protein 10), *Erbb4* (v-erb-a erythroblastic leukemia viral oncogene homolog 4), *Irx4* (Iroquois related homeobox 4), and *Atp1a2* (ATPase, Na^+/K^+ transporting, α 2 polypeptide) (Table S2). We also identified genes that were expressed to a greater extent in both cardiomyocytes and 4 week iCMs than in fibroblasts (group 2 in

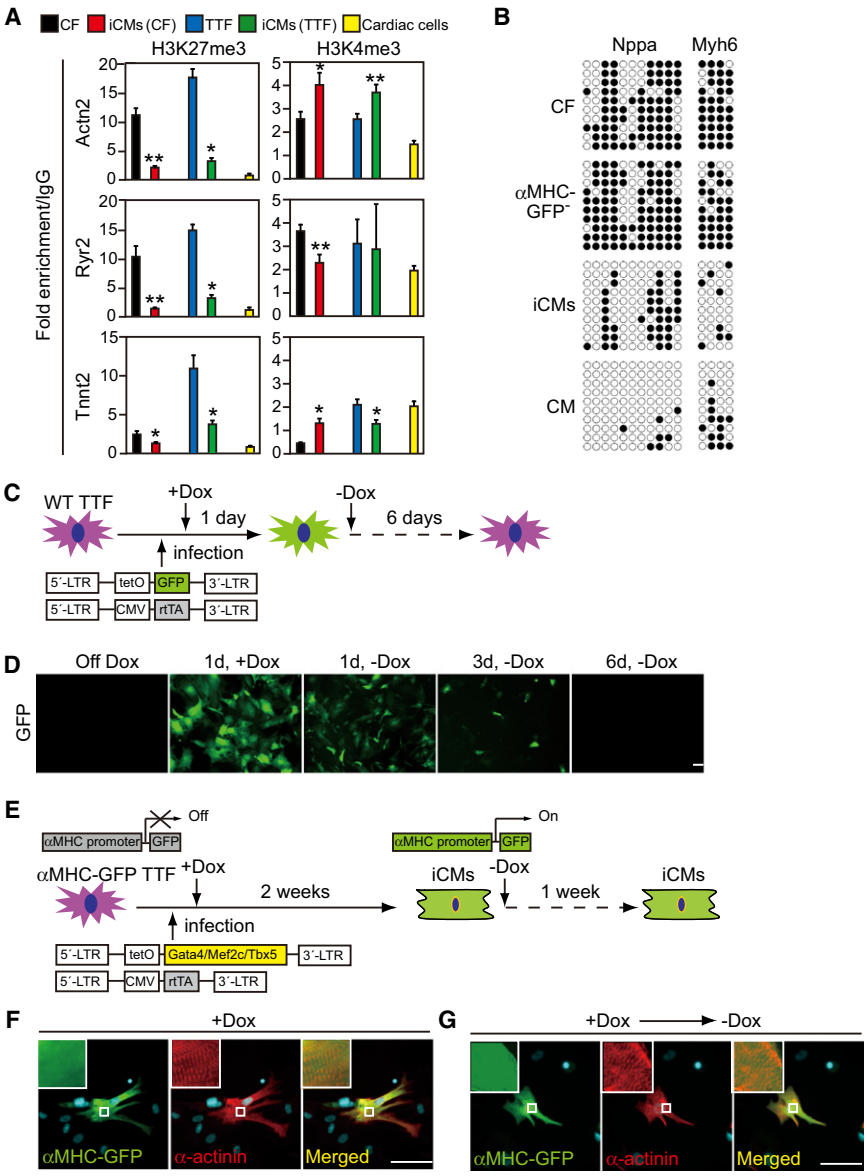

Figure 5. Fibroblasts Are Stably Reprogrammed into iCMs by Gata4, Mef2c, and Tbx5

(A) The promoters of *Actn2*, *Ryr2*, and *Tnnt2* were analyzed by ChIP for trimethylation status of histone H3 of lysine 27 or 4 in cardiac fibroblasts (CF), CF-derived iCMs, tail-tip fibroblasts (TTF), TTF-derived iCMs, and neonatal cardiac cells. Data were quantified by qPCR.

(B) The promoters of *Nppa* and *Myh6* were analyzed with bisulfite genomic sequencing for DNA methylation status in CF, α-MHC-GFP$^-$ cells, α-MHC-GFP$^+$ iCMs (FACS sorted 4 weeks after transduction), and neonatal CM. Open circles indicate unmethylated CpG dinucleotides; closed circles indicate methylated CpGs.

(C) Schematic representation of the strategy to test expression kinetics of the doxycycline (Dox)-inducible lentiviral system.

(D) Wild-type TTFs were infected with pLVX-tetO-GFP and pLVX-rtTA and imaged before (off Dox), 1 day after Dox addition, and at time points after Dox withdrawal (–Dox). All images were taken using constant exposure times and identical camera settings.

(E) Schematic representation of the strategy to determine temporal requirement of Gata4/Mef2c/Tbx5 in reprogramming. Thy1$^+$/GFP$^-$ TTF were infected with the pLVX-tetO-GMT and pLVX-rtTA lentiviruses, and Dox was added for 2 weeks and thereafter withdrawn for 1 week.

(F) Immunofluorescent staining for GFP, α-actinin, and DAPI in iCMs 2 weeks after lentiviral infection and Dox induction.

(G) Immunofluorescent staining for GFP, α-actinin, and DAPI 1 week after Dox withdrawal. iCMs maintained α-MHC GFP expression and had α-actinin positive sarcomeric structures. High-magnification views in insets show sarcomeric organization. Representative data are shown in each panel. All data are presented as means ± SD. *$p < 0.01$; **$p < 0.05$ versus relevant control. Scale bars represent 100 μm.

Figure 4E), including *Actc1*, *Myl7* (myosin, light polypeptide 7, regulatory, also known as MLC2a), *Tnnt2* (troponin T2, cardiac), *Tbx3* (T-box 3), and *Srf* (serum response factor) (Table S2). Thus, iCMs were similar, but not identical, to neonatal cardiomyocytes, and the reprogramming event was broadly reflected in global gene expression changes.

Fibroblasts Are Epigenetically Reprogrammed to a Cardiomyocyte-like State by Gata4/Mef2c/Tbx5

To determine if iCMs have gained a cardiomyocyte-like chromatin state, we analyzed the enrichment of histone modifications in the promoter regions of the cardiac-specific genes *Actn2*, *Ryr2l*, and *Tnnt2*. We analyzed the enrichment of trimethylated histone H3 of lysine 27 (H3K27me3) and lysine 4 (H3K4me3), which mark transcriptionally inactive or active chromatin, respectively (Li et al., 2007), in cardiac fibroblasts, 4 week iCMs, and neonatal cardiac cells by chromatin immunoprecipitation, followed by qPCR (Figure 5A). After reprogramming, H3K27me3 was significantly depleted at the promoters of all the genes analyzed in iCMs, reaching levels comparable to those in cardiac cells, whereas H3K4me3 increased on the promoter regions of *Actn2* and *Tnnt2* in iCMs, as compared with cardiac fibroblasts. *Ryr2* had similar levels of H3K4me3 in iCMs as in fibroblasts, suggesting that its activation reflects the resolution of a "bivalent" chromatin mark (Bernstein et al., 2006). These results suggested that cardiac fibroblast-derived iCMs gained a chromatin status similar to cardiomyocytes at least in some cardiac specific genes. Intriguingly, H3K27me3 levels were higher in tail-tip fibroblasts than cardiac fibroblasts on all three genes analyzed and, despite a significant reduction upon reprogramming to iCMs, remained somewhat higher than in cardiac cells and cardiac fibroblast-derived iCMs.

The DNA methylation status of specific loci also reflects the stability of the reprogramming event and we therefore investigated such changes during reprogramming from cardiac fibroblasts to iCMs. We performed bisulfite genomic sequencing in the promoter regions of *Nppa* and *Myh6* in cardiac fibroblasts, 4 week GFP$^-$ cells, iCMs, and neonatal cardiomyocytes. Both promoter regions were hypermethylated in cardiac fibroblasts and GFP$^-$ cells, as expected from the cardiomyocyte-specific expression of these genes, but were comparatively demethylated in iCMs, similar to neonatal cardiomyocytes (Figure 5B). These results indicated that reprogramming by Gata4, Mef2c, and Tbx5 induced epigenetic resetting of the fibroblast genome to a cardiomyocyte-like state.

To further assess the stability of the reprogramming event, we generated a doxycycline-inducible lentiviral system in which transgene expression of the reprogramming factors was controlled by doxycycline administration. We first transduced wild-type tail-tip fibroblasts with a mixture of lentiviruses containing pLVX-tetO-GFP and pLVX-rtTA to determine the expression kinetics of this system (Figure 5C). We confirmed that the majority of fibroblasts infected with both viruses expressed GFP within 1 day after doxycycline induction, and the GFP expression was instantly diminished by withdrawal of doxycycline and disappeared within 6 days (Figure 5D). Thy1$^+$/GFP$^-$ tail-tip fibroblasts were harvested from αMHC-GFP neonatal mice, transduced with a pool of lentiviruses containing inducible *Gata4*, *Mef2c*, and *Tbx5*, along with pLVX-rtTA, and subsequently treated with doxycycline (Figure 5E). We found that αMHC-GFP expression was induced from tail-tip fibroblasts after doxycycline administration and that the iCMs had well-defined sarcomeric structures marked with an anti-α-actinin antibody after 2 weeks of culture (Figure 5F). Doxycycline was withdrawn after 2 weeks of culture, and cells were subsequently cultured without doxycycline for 1 week to fully remove exogenous expression of the reprogramming factors (Figure 5E). The iCMs maintained αMHC-GFP expression and had sarcomeric structures after doxycycline withdrawal, suggesting that the fibroblasts were stably reprogrammed into iCMs after 2 weeks exposure to Gata4, Mef2c, and Tbx5 (Figure 5G).

Induced Cardiomyocytes Exhibit Spontaneous Contraction

To determine if iCMs possessed the functional properties characteristic of cardiomyocytes, we analyzed intracellular Ca^{2+} flux in iCMs after 2–4 weeks of culture. Around 30% of cardiac fibroblast-derived iCMs showed spontaneous Ca^{2+} oscillations and their frequency was variable, resembling what was observed in neonatal cardiomyocytes (Figures 6A, 6B, and 6D; Movie S1). We observed that tail-tip dermal fibroblast-derived iCMs also exhibited spontaneous Ca^{2+} oscillations, but the oscillation frequency was lower than that of cardiomyocytes and cardiac fibroblast-derived iCMs (Figures 6C and 6E; Movie S2).

In addition to the characteristic Ca^{2+} flux, cardiac fibroblast-derived iCMs showed spontaneous contractile activity after 4–5 weeks in culture (Movies S3 and S4; Figure S5). Single-cell extracellular recording of electrical activity in beating cells revealed tracings similar to the potential observed in neonatal cardiomyocytes (Figure 6F). Intracellular electrical recording of iCMs displayed action potentials that resembled those of adult mouse ventricular cardiomyocytes (Figure 6G). Thus, the reprogramming of fibroblasts to iCMs was associated with global changes in gene expression, epigenetic reprogramming, and the functional properties characteristic of cardiomyocytes.

Transplanted Cardiac Fibroblasts Transduced with Gata4/Mef2c/Tbx5 Reprogram In Vivo

To investigate whether GMT-transduced cardiac fibroblasts can be reprogrammed to express cardiomyocyte-specific genes in their native environment in vivo, we harvested GFP$^-$/Thy1$^+$ cardiac fibroblasts 1 day after viral transduction and injected them into immunosuppressed NOD-SCID mouse hearts. GMT-infected cells did not express GFP at the time of transplantation (Figure 4A). Cardiac fibroblasts were infected with either the mixture of GMT and DsRed retroviruses or DsRed retrovirus (negative control) to be readily identified by fluorescence. Cardiac fibroblasts infected with DsRed did not express α-actinin or GFP, confirming cardiomyocyte conversion did not happen in the negative control (Figures 7A and 7B). Despite being injected into the heart only 1 day after viral infection, a subset of cardiac fibroblasts transduced with GMT and DsRed expressed GFP in the mouse heart within 2 weeks (Figure 7B). Importantly, the GFP$^+$ cells expressed α-actinin and had sarcomeric structures (Figure 7C). These results suggested that cardiac fibroblasts transduced with *Gata4*, *Mef2c*, and *Tbx5* can reprogram to cardiomyocytes within 2 weeks upon transplantation in vivo.

DISCUSSION

Here we demonstrated that the combination of three transcription factors, Gata4, Mef2c, and Tbx5, can rapidly and efficiently induce cardiomyocyte-like cells from postnatal cardiac and dermal fibroblasts. iCMs were similar to neonatal cardiomyocytes in global gene expression profile, electrophysiologically, and could contract spontaneously, demonstrating that functional cardiomyocytes can be generated from differentiated somatic cells by defined factors. Although much refinement and characterization of the reprogramming process will be necessary, the findings reported here raise the possibility of reprogramming the vast pool of endogenous fibroblasts that normally exists in the heart into functional cardiomyocytes for regenerative purposes.

The three reprogramming factors, Gata4, Mef2c, and Tbx5, are core transcription factors during early heart development (Olson, 2006; Srivastava, 2006; Zhao et al., 2008). They interact with one another, coactivate cardiac gene expression (e.g., *Nppa*, *Gja5* [Cx40], and *Myh6*), and promote cardiomyocyte differentiation (Bruneau et al., 2001; Garg et al., 2003; Ghosh et al., 2009; Lin et al., 1997). Gata4 is considered a ''pioneer'' factor and might open chromatin structure in cardiac loci (Cirillo et al., 2002), thus allowing binding of Mef2c and Tbx5 to their specific target sites and leading to full activation of the cardiac program. Although the reprogramming event appears stable at the epigenetic level, as marked by histone methylation and DNA methylation, the global gene expression of iCMs is similar but not identical to neonatal cardiomyocytes. Whether they are

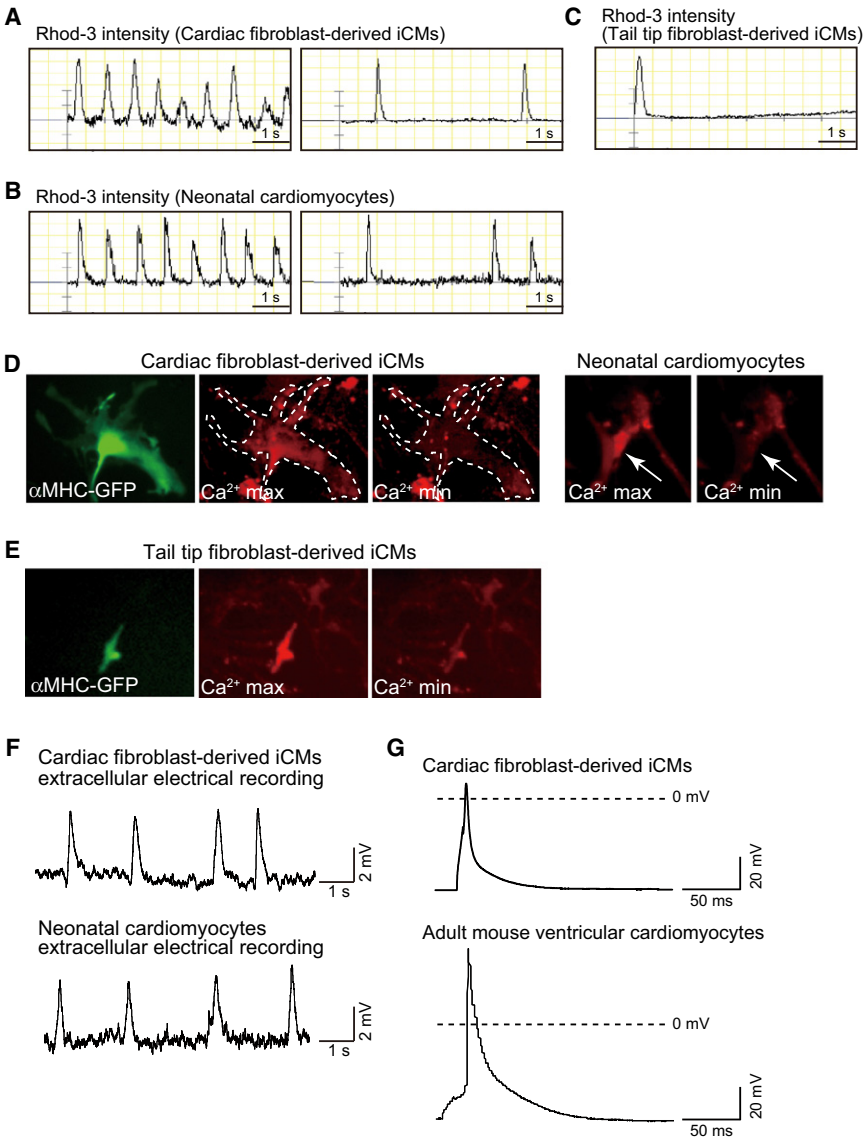

Figure 6. Induced Cardiomyocytes Exhibit Spontaneous Ca^{2+} Flux, Electrical Activity, and Beating

(A and B) Cardiac fibroblast (CF)-derived iCMs showed spontaneous Ca^{2+} oscillation with varying frequency (A), similar to neonatal cardiomyocytes (B). Rhod-3 intensity traces are shown.

(C) Tail-tip dermal fibroblast (TTF)-derived iCMs showed spontaneous Ca^{2+} oscillation with lower frequency. The Rhod-3 intensity trace is shown.

(D) Spontaneous Ca^{2+} waves observed in CF-derived α-MHC-GFP$^+$ iCMs (white dots) or neonatal cardiomyocytes (arrows) with Rhod-3 at Ca^{2+} max and min is shown. Fluorescent images correspond to the Movie S1.

(E) Spontaneous Ca^{2+} oscillation observed in the TTF-derived α-MHC-GFP$^+$ iCMs with Rhod-3 at Ca^{2+} max and min is shown. Fluorescent images correspond to the Movie S2.

(F) Spontaneously contracting iCMs had electrical activity measured by single cell extracellular electrodes. Neonatal cardiomyocytes showed similar electrical activity.

(G) Intracellular electrical recording of CF-derived iCMs cultured for 10 weeks displayed action potentials that resembled those of adult mouse ventricular cardiomyocytes. Representative data are shown in each panel (n = 10 in A–F, n = 4 in G). See also Figure S5 and Movies S1, S2, S3 and S4.

See also Movies S1, S2, S3, and S4 and Figure S5.

more similar to adult ventricular cardiomyocytes or other subpopulations remains to be determined. Additional epigenetic regulators, microRNAs, or signaling proteins may be leveraged to increase the efficiency and robustness of the reprogramming event. Furthermore, other combinations of factors likely also induce cardiac reprogramming, much like the experience in the iPSC field.

Several lines of evidence suggest that the iCMs we describe here originated from differentiated fibroblasts. We found that any potential rare cardiac "progenitor-like" cells, marked by c-kit or Isl1, were dispensable for cardiomyocyte induction (Beltrami et al., 2003). Furthermore, the high efficiency of cardiac induction (up to 20%) does not favor the interpretation that rare stem or progenitor cells were the origin of induced cardiomyocytes. Most importantly, the ability to reprogram dermal fibroblasts into iCMs supports the conclusion that cardiac progenitors are not the target cells for the reprogramming factors. Remarkably, reprogramming of cardiac fibroblasts to myocytes occurred in a relatively short period, with the first GFP$^+$ cells appearing at day 3, in contrast to iPSC reprogramming, which typically takes 10–20 days and occurs with much lower efficiency (<0.1%) (Takahashi and Yamanaka, 2006). Despite the early initiation of reprogramming, the process appears to continue for several weeks, with progressive changes in gene expression, contractile ability, and electrophysiologic maturation.

Although many questions remain regarding the mechanisms of reprogramming, we were able to genetically test the "route" of cell fate alteration. Our findings suggest that cardiomyocytes were directly induced from cardiac fibroblasts without reverting to a cardiac progenitor cell state, which may explain the rapid early reprogramming process. This conclusion was supported by the absence of Isl1-Cre-YFP or Mesp1-Cre-YFP activation during the process of reprogramming, which would have marked any cells that transiently expressed Isl1 or Mesp1 (Laugwitz et al., 2005; Saga et al., 1999).

The ability to reprogram endogenous cardiac fibroblasts into cardiomyocytes has many therapeutic implications. First, the avoidance of reprogramming to pluripotent cells before cardiac differentiation would greatly lower the risk of tumor formation in the setting of future cell-based therapies. Second, large

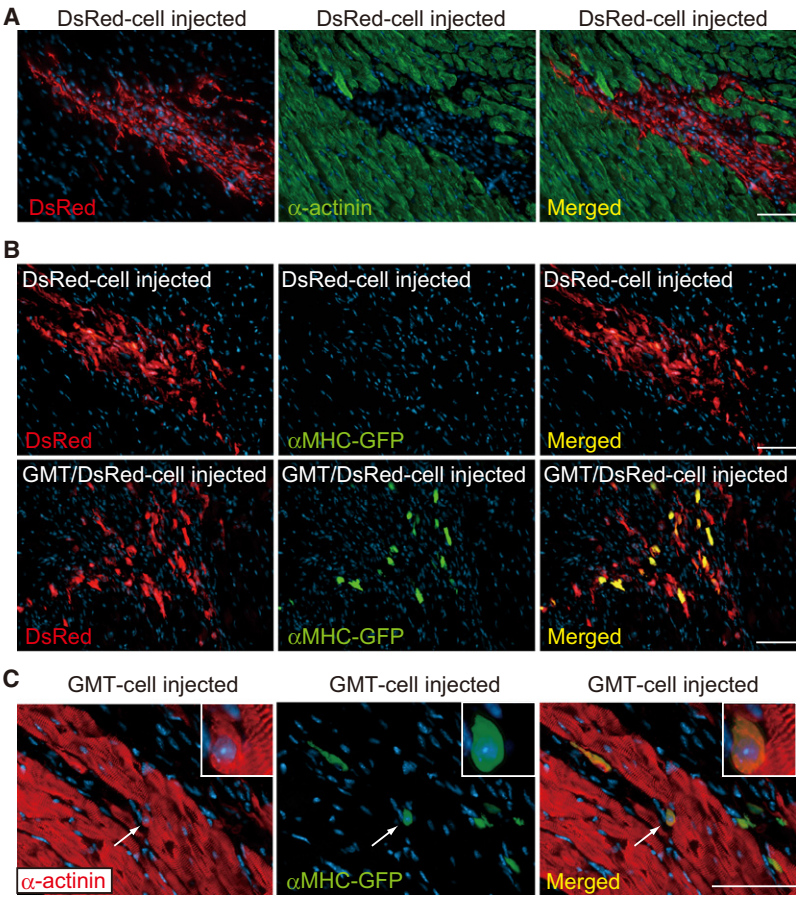

Figure 7. Transplanted Cardiac Fibroblasts Transduced with Gata4/Mef2c/Tbx5 Can Be Reprogrammed to Cardiomyocytes In Vivo

(A) DsRed infected cardiac fibroblasts (DsRed-cell) were transplanted into NOD-SCID mouse hearts 1 day after infection and cardiac sections were analyzed by immunocytochemistry after 2 weeks. Transplanted fibroblasts marked with DsRed did not express α-actinin (green).

(B) Cardiac fibroblasts infected with DsRed or Gata4/Mef2c/Tbx5 with DsRed (GMT/DsRed-cell) were transplanted into NOD-SCID mouse hearts 1 day after infection and visualized by histologic section. Note that a subset of GMT/DsRed cells expressed α-MHC-GFP. Data were analyzed 2 weeks after transplantation.

(C) Gata4/Mef2c/Tbx5-transduced cardiac fibroblasts (GMT-cell) were transplanted into mouse hearts and histologic sections analyzed. A subset of induced GFP$^+$ cells expressed α-actinin (red) and had sarcomeric structures. Insets are high-magnification views of cells indicated by arrows. Data were analyzed 2 weeks after transplantation. Representative data are shown in each panel (n = 4 in each group). Scale bars represent 100 μm. Note that GMT/DsRed or GMT-infected cells did not express GFP at the time of transplantation (Figure 4A).

amounts of an individual's own fibroblasts can be grown from a cardiac biopsy or skin biopsy in vitro for transduction with the defined factors, followed by delivery of cells to damaged hearts. Third, and most promising, is the potential to introduce the defined factors, or factors that mimic their effects, directly into the heart to reprogram the endogenous fibroblast population, which represents more than 50% of the cells, into new cardiomyocytes that can contribute to the overall contractility of the heart. Our observation that injection of fibroblasts into the heart only 1 day after induction of Gata4/Mef2c/Tbx5 resulted in reprogramming of the transplanted cells suggests that this may be possible. Future studies in human cells and advances in safe delivery of defined factors will be necessary to advance this technology for potential regenerative therapies.

EXPERIMENTAL PROCEDURES

Generation of αMHC-GFP, Isl1-YFP, and Mesp1-YFP Mice

To generate αMHC-GFP mice, EGFP-IRES-Puromycin cDNA was subcloned into the expression vector containing α-myosin heavy chain promoter (Gulick et al., 1991). Pronuclear microinjection and other procedures were performed according to the standard protocols (Ieda et al., 2007). PCR primers are listed in the Extended Experimental Procedures. Isl1-YFP mice were obtained by crossing Isl1-Cre mice and R26R-EYFP mice (Srinivas et al., 2001). Mesp1-YFP mice were obtained by crossing Mesp1-Cre mice and R26R-EYFP mice (Saga et al., 1999).

Cell Culture

For explant culture, isolated neonatal or adult mouse hearts were minced into small pieces less than 1 mm^3 in size. The explants were plated on gelatin-coated dishes and cultured for 7 days in explant medium (IMDM/20% FBS) (Andersen et al., 2009). Migrated cells were harvested and filtered with 40 μm cell strainers (BD) to avoid contamination of heart tissue fragments. αMHC-GFP$^-$/Thy1$^+$, Isl1-YFP$^-$/Thy1$^+$, αMHC-GFP$^-$/Thy1$^+$/c-kit$^-$, or αMHC-GFP$^-$/Thy1$^+$/c-kit$^+$ live cells (as defined by the lack of propidium iodine staining) were isolated using FACS Aria 2 (BD Biosciences). For conventional isolation of neonatal cardiac fibroblasts, hearts were digested with 0.1% trypsin and plated on plastic dishes (Ieda et al., 2009). For isolation of tail-tip fibroblasts, tails were digested with 0.1% trypsin and plated on plastic dishes. Attached fibroblasts were cultured for 7 days and αMHC-GFP$^-$/Thy1$^+$ or Mesp1-YFP$^-$/Thy1$^+$ cells were sorted and cultured in DMEM/M199 medium containing 10% FBS at a density of 10^4/cm^2. Cells were transduced by retroviruses or lentiviruses after 24 hr.

Isolation of Cardiomyocytes

To isolate cardiomyocytes, neonatal αMHC-GFP$^+$ ventricles were cut into small pieces and digested with collagenase type II solution (Ieda et al., 2009). A single-cell suspension was obtained by gentle triturating and passing through a 40 μm cell strainer. αMHC-GFP$^+$ live cells were isolated by FACS Aria 2. To obtain cardiac cells, cells were plated on gelatin-coated plastic dishes and treated with Ara C (Sigma) to inhibit nonmyocyte proliferation.

Molecular Cloning and Retroviral/Lentiviral Infection

Retroviruses or inducible lentiviruses containing the cardiac developmental factors were generated as described and as detailed in the Extended Experimental Procedures (Kitamura et al., 2003; Takahashi and Yamanaka, 2006). The pMXs-DsRed Express retrovirus infection in cardiac fibroblasts resulted in >95% transduction efficiency (Hong et al., 2009).

FACS Analyses and Sorting

For GFP expression analyses, cells were harvested from cultured dishes and analyzed on a FACS Calibur (BD Biosciences) with FlowJo software. For αMHC-GFP/cTnT expression, cells were fixed with 4% PFA for 15 min, permeabilized with Saponin, and stained with anti-cTnT and anti-GFP antibodies,

followed by secondary antibodies conjugated with Alexa 488 and 647 (Kattman et al., 2006).

For αMHC-GFP$^-$/Thy1$^+$, Isl1-YFP$^-$/Thy1$^+$, and Mesp1-YFP$^-$/Thy1$^+$ cell sorting, cells were incubated with PECy7 or APC-conjugated anti-Thy1 antibody (eBioscience) and sorted by FACS Aria 2 (Ieda et al., 2009). For αMHC-GFP$^-$/Thy1$^+$/c-kit$^-$ and αMHC-GFP$^-$/Thy1$^+$/c-kit$^+$ cell sorting, PECy7-conjugated anti-Thy1 and APC-conjugated anti-c-kit antibodies (BD) were used. We used bone marrow cells as a positive control for c-kit staining.

Cell Transplantation

Fibroblasts were harvested 1 day after retroviral infection. A left thoracotomy was carried out in NOD-SCID mice, and 10^6 cultured cells were injected into the left ventricle. After 1–2 weeks, the hearts were excised for immunohistochemistry.

Histology and Immunocytochemistry

Cells or tissues were fixed, processed and stained with antibodies against numerous proteins in standard fashion as detailed in the Extended Experimental Procedures.

Quantitative RT-PCR

Total RNA was isolated from cells, and qRT-PCR was performed on an ABI 7900HT (Applied Biosystems) with TaqMan probes (Applied Biosystems), which are listed in the Extended Experimental Procedures. To quantify endogenous-specific transcripts and both endogenous and transgene common transcripts, primers were designed using Vector NTI, and SYBR green technology was used. Primer information is available on request. mRNA levels were normalized by comparison to *Gapdh* mRNA.

Microarray Analyses

Mouse genome-wide gene expression analyses were performed using Affymetrix Mouse Gene 1.0 ST Array. αMHC-GFP$^+$ cardiomyocytes were collected by FACS. Three-factor transduced GFP$^+$ cells and GFP$^-$ cells were collected by FACS after 2 and 4 weeks of culture. Cardiac fibroblasts were also collected after 4 weeks of culture. RNA was extracted using PicoPure RNA Isolation (Arcturus). Microarray analyses were performed in triplicate from independent biologic samples, according to the standard Affymetrix Genechip protocol. Data were analyzed using the Affymetrix Power Tool (APT, version 1.8.5). See the Extended Experimental Procedures for additional statistical methods.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitations were performed on cardiac fibroblasts, tail-tip dermal fibroblasts, iCMs, and neonatal cardiac cells. Immunoprecipitations were done using the Imprint Chromatin Immunoprecipitation Kit (Sigma) following the manufacturer instructions. Antibodies against H3K27me3 and H3K4me3 were from Active motif, and normal rabbit IgG was from Cell Signaling Technology. Primer sequences for qPCR custom TaqMan gene expression assays (Applied Biosystems) are listed in the Extended Experimental Procedures.

Bisulfite Genomic Sequencing

Bisulfite treatment was performed using the Epitect Bisulfite Kit (QIAGEN) according to the manufacturer's recommendations. PCR primers are listed in the Extended Experimental Procedures. Amplified products were cloned into pCR2.1-TOPO (Invitrogen). Ten randomly selected clones were sequenced with the M13 forward and M13 reverse primers for each gene.

Ca^{2+} Imaging

Ca^{2+} imaging was performed according to the standard protocol. Briefly, cells were labeled with Rhod-3 (Invitrogen) for 1 hr at room temperature, washed, and incubated for an additional 1 hr to allow de-esterification of the dye. Rhod-3-labeled cells were analyzed by Axio Observer (Zeiss) with MiCAM02 (SciMedia).

Electrophysiology

After 4 week transduction with GMT, the electrophysiological activities of iCMs were analyzed using extracellular electrode recording with an Axopatch 700B amplifier and the pClamp9.2 software (Axon Instruments). iCMs were visually identified by GFP expression and spontaneous contraction. Glass patch pipettes, with typical resistances of 2–4 MΩ, were directly attached on single GFP$^+$ cells for extracellular recording in Tyrode's bath solution. For recording intracellular action potentials, single GFP$^+$ cells were held at −70 mV membrane potential with a stimulation of 0.1–0.5 nA for 5 ms to elicit a response after 10-week transduction with GMT.

Statistical Analyses

Differences between groups were examined for statistical significance using Student's t test or ANOVA. p values of <0.05 were regarded as significant.

ACCESSION NUMBERS

Microarray data have been submitted and can be accessed by the Gene Expression Omnibus (GEO) accession number GSE22292.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, two tables, and four movies and can be found with this article online at doi:10.1016/j.cell.2010.07.002.

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